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N-cadherin regulates signaling mechanisms required for lens fiber cell elongation and lens morphogenesis

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Abstract

Tissue development and regeneration involve high-ordered morphogenetic processes that are governed by elements of the cytoskeleton in conjunction with cell adhesion molecules. Such processes are particularly important in the lens whose structure dictates its function. Studies of our lens-specific N-cadherin conditional knockout mouse (N-cadcKO) revealed an essential role for N-cadherin in the migration of the apical tips of differentiating lens fiber cells along the apical surfaces of the epithelium, a region termed the Epithelial Fiber Interface (EFI), that is necessary for normal fiber cell elongation and the morphogenesis. Studies of the N-cadcKO lens suggest that N-cadherin function in fiber cell morphogenesis is linked to the activation of Rac1 and myosin II, both signaling pathways central to the regulation of cell motility including determining the directionality of cellular movement. The absence of N-cadherin did not disrupt lateral contacts between fiber cells during development, and the maintenance of Aquaporin-0 and increased expression of EphA2 at cell-cell interfaces suggests that these molecules may function in this role. E-cadherin was maintained in newly differentiating fiber cells without interfering with expression of lens-specific differentiation proteins but was not able to replace N-cadherin function in these cells. The dependence of migration of the fiber cell apical domains along the EFI for lens morphogenesis on N-cadherin provides new insight into the process of tissue development.

Keywords

N-cadherin; lens; morphogenesis; tissue development

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Introduction

Tissue morphogenesis is a highly-ordered process dependent on individual cells acquiring morphologies that define their differentiated function and the organization of these differentiating cells into a mature tissue structure. The lens is a tissue in which structure defines function, and has emerged as an ideal model for studying how differentiating cells are guided to organize a functional tissue. The cytoarchitecture of the lens is highly ordered and dominated by a single differentiated cell type, the elongated lens fiber cell. As fiber cells elongate they surround the cells that have differentiated before them and arrange laterally with their near neighbors into a hexagonally packed cellular array. Precise morphogenetic patterning is central to lens transparency and the function of the lens of focusing incoming light signals onto the retina.

Establishing the unique cytoarchitecture of the lens during development involves a series of highly coordinated morphogenetic events dependent, in part, on signaling inputs from BMPs, FGFs, IGF-1, and Jagged/Notch (Bassas et al., 1987; Le and Musil, 2001; Walker et al., 2002; Rowan et al., 2008; Lovicu et al., 2011; Huang et al., 2015; Kalcheim, 2015). However, creating and maintaining lens cytoarchitecture also depends greatly on structural inputs, such as from cell-cell junctions and their associated cytoskeletal/signaling networks (Menko, 2002; Rao and Maddala, 2006; Maddala et al., 2007; Leonard et al., 2011; Maddala et al., 2011; Maddala et al., 2015) whose function in the regulation of lens development is less well understood. Members of the cadherin family of cell-cell adhesion receptors have been implicated as molecular regulators of lens morphogenesis. E-cadherin and N-cadherin are essential for separation of the initial lens vesicle from head ectoderm during early lens development (Pontoriero et al., 2009). Both are expressed by undifferentiated lens epithelial cells throughout development (Leonard et al., 2011; Leonard et al., 2013; Maddala et al., 2015), but E-cadherin expression is blocked after fiber cell differentiation has begun. N-cadherin junctions then organize along fiber cell lateral interfaces coordinately with cortical F-actin (Leonard et al., 2011). As newly differentiating fiber cells turn at the lens fulcrum they begin to elongate, their apical surfaces associating with and migrating along the apical surfaces of cells in the adjacent lens epithelium, creating a region defined as the Epithelial-Fiber Interface (EFI). The fiber cells continue on this migration path until their apical tips reach the central-most epithelium where they form the anterior lens sutures. With the concurrent migration of their basal surfaces along the posterior lens capsule, highly elongated, convex shaped fiber cells are continually added to the growing lens during development.

While loss of N-cadherin junctions is a common feature of knockouts or mutations causing lens dymorphogenesis (Cooper et al., 2008; Cheng and Gong, 2011; Son et al., 2012; Cheng et al., 2013; Biswas et al., 2016), the mechanisms by which N-cadherin junctions function as regulators of normal lens morphogenesis has remained elusive. Deletion of N-cadherin at the placode stage from all Pax6-expressing eye tissues causes considerable lens deterioration by P21, but despite their smaller size, the lenses in the LeCre-induced N-cadherin knockout mouse develop normally and form distinct epithelial and fiber cell compartments (Pontoriero et al., 2009). Altered ZO-1 and Aquaporin-0 (Aqp0) localization in these lenses suggested there might be a defect in fiber cell elongation. To examine N-cadherin function specifically

in the morphogenetic differentiation of lens fiber cells we created a lens-specific N-cadherin conditional knockout mouse (N-cadcKO) in which N-cadherin is present during lens vesicle formation and eliminated as primary lens fiber cells begin to differentiate. Our studies with these N-cadcKO lenses revealed that N-cadherin was required for the migration of the apical tips of newly differentiating fiber cells along the EFI, and that this mechanism was required for normal fiber cell elongation and lens morphogenesis.

Methods

Lens-specific N-cadherin conditional knockout (N-cadcKO) mice

Previously generated N-cadherin fl/fl mice on a mixed 129Sv/C57Bl/6J genetic background (Kostetskii et al., 2005; Li et al., 2005; Piven et al., 2011) were mated with αA-crystallin/Cre mice (MLR10) (Zhao et al., 2004) on a FVB/N background that lacks expression of filensin and CP49 (Simirskii et al., 2007). This mixed genetic background ensured expression of filensin and CP49 in N-cadherin conditional knockout lenses, which was confirmed by protein analysis. Cre expression in MLR10cre mice begins at 10.5, when it is limited to the differentiating, primary lens fiber cells, and eventually spreads to all lens cells. Mice were genotyped for N-cadherin and Cre by PCR as previously described (Rivera et al., 2009; Piven et al., 2011). The N-cadherin floxed allele was detected with the following primers: L06- 5′-CCAAAGCTGAGTGTGACTTG-3′, L08- 5′-TACAAGTTTGGGTGACAAGC, and the Cre transgene detected using the primers: PR4-5′-GCATTCCAGCTGCTGACGGT-3′, CRE-AS-5′-CAGCCCGGACCGACGATGAAG-3′. Littermates of the following genotypes were used: MLR10/Cre; N-cadherin f/f (Ncad KO) and N-cadherin f/f (minus Cre, N-cadherin WT) as control. Animal experiments were performed in accordance with IACUC guidelines of Thomas Jefferson University and guidelines of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Gestational age was determined through detection of a vaginal plug, with day 0.5 (E0.5) of embryogenesis defined as noon of the day of the appearance of the plug. While penetrance of the N-cadcKO was 100%, there was some variability in severity of the observed morphogenetic defect. In each study presented we compare littermates and present results representative of the phenotype.

Immunostaining

Isolated mouse eyes were fixed in 3.7% formaldehyde overnight at 4 °C, cryoprotected in 30% sucrose solution for a minimum of 24 h prior to freezing and 20-μm thick cryosections cut. Sections were incubated in 0.25% Triton X-100 in DPBS buffer (2.7 mM KCl, 1.5 mM KH2PO4, 137.9 mM NaCl, 8.1 mM Na2HPO4–7 H2O [Corning, 21-0310CV]) for 10 min, followed by blocking buffer (5% goat serum, 0.5 g BSA in 50 ml DPBS) for 1 h prior to labeling. Samples were incubated sequentially in primary antibody at 4 °C overnight, followed by fluorescent-conjugated secondary antibody for 1 h at 37°C (Jackson ImmunoResearch Laboratories, 111-295-144, 115-545-003, 115-295-008). Primary antibodies used included: N-cadherin (BD Bioscience, 610921), β-catenin (BD Bioscience, 610154), E-cadherin (Cell Signaling, 24E10), βB-crystallin (Santa Cruz, FL-252), Rac1 (Abcam, ab155938), myosin-II (LifeSpan BioSciences, LS-C84042), WGA (LifeSpan

BioSciences, LS-C76576), ZO-1 (Abcam, 61357), EphA2 (R&D, AF639), connexin 50 (ADI, Cx50-A), aquaporin-0 (ADI, AQP01-A), active Rac1 (NewEast Biosciences, 26903), p-myosin (Cell Signaling, 3674), and MLCK (Abcam, ab76092). F-actin was localized with Alexa448-conjugated phalloidin (Invitrogen-Molecular Probes). Nuclei were labeled with TO-PRO-3 (Invitrogen-Molecular Probes).

Image Analysis

Confocal microscopy was performed using a Zeiss LSM510META confocal microscope. Z-stacks were collected, and single optical planes (1.0 μm) selected using the LSM Image Browser.

Immunoblotting

A Triton X-100/Octylglucoside (OG/T) buffer cocktail composed of 44.4 mM *n*-octyl β-D-glucopyranoside, 1% Triton X-100, 100 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA, and 10 mM imidazole, containing 1 mM sodium vanadate, 0.2 mM H_2O_2, and protease inhibitor cocktail (Sigma Aldrich, P8340) was used to extract E16.5 wildtype and N-cadcKO lenses isolated from embryos. For studies of phospho-myosin and active Rac1, Halt™ Phosphatase Inhibitor (Thermo Scientific #1862495) and Protease/Phosphatase Inhibitor Cocktail (Cell Signaling 5872S) were added to extraction buffer. The concentration of protein was determined using the BCA assay (Thermo Scientific, 23223, 23224). 15–30 μg of protein extracts were subjected to SDS-PAGE on precast 8–16% Tris/glycine gels (Invitrogen, EC6045BOX). Proteins were electrophoretically transferred onto Immobilon-P membranes (Millipore Corp., IPVH00010). Membranes were blocked in 5% skim milk for 1 h and probed for primary antibodies at 4 °C overnight followed by secondary antibodies conjugated to horseradish peroxidase (BIO-RAD, 170-6515, 170-6516). Primary antibodies used included: N-cadherin (BD Bioscience, 610921), βB-crystallin (Santa Cruz, FL-252), Rac1 (Abcam, ab155938), myosin-II (LifeSpan BioSciences, LS-C84042), EphA2 (R&D, AF639), connexin 50 (ADI, Cx50-A), aquaporin-0 (ADI, AQP01-A), active Rac1 (NewEast Biosciences, 26903), p-myosin (Cell Signaling, 3674). Protein bands were detected using ECL reagent or ECL plus reagent (Thermo Fisher Scientific Inc. Rockford, 32106, 80197) and images were acquired using the FluorChem E & M imager from Protein Simple (FM0418). The FluorChem E & M imager is a digital darkroom technology with an 8.3 megapixel CCD (charge-coupled device) resolution and flat field calibration that corrects for non-uniformities in light gathering. The densitometric analysis of immunoblots was performed using Alpha View® software (Protein Simple). Representative blots were used for figures. Immunoblot bands were quantified, ratioed to GAPDH (loading control), and then normalized to wildtype values. Graphs depict average values for N-cadcKO immunoblots and error bars represent SEM. Statistical analysis was performed using *t* test on 3 or more independent experiments comparing normalized wild-type values to N-cadcKO values using the SPSS statistics software. Differences were considered significant when $*P \leq 0.05$, $**P \leq 0.01$ and, $***P \leq 0.001$.

Lens Measurements

Lens height and width measurement were performed using LSM Image Browser and Adobe Photoshop. Lens area was then calculated using the formula for an ellipse. To calculate

average secondary fiber cell width, individual fiber cells equidistant from the lens fulcrum were measured using Adobe Photoshop and averaged across multiple lenses, taken from the middle section of wildtype and N-cadcKO lenses.

Immunostaining Intensity Measurements

ImageJ Analysis Software was used to import Zeiss LSM510META confocal microscope images. Representative areas measuring 200μm × 200μm from both the epithelium and fiber cell zones of wildtype and N-cadcKO lenses were outlined to generate pixel intensity value plots from which image histogram readouts were generated.

Results

Dynamics of cadherin junctions during lens morphogenesis

The first stage of lens differentiation begins early in development after the lens placode pinches off from head ectoderm as a hollow vesicle of epithelial cells. Its posterior epithelial cells elongate coordinately to form primary fibers, taking a direct linear pathway towards the lens anterior. In the developing mouse lens, the apical tips of these fiber cells complete their elongation by E13.5. Their point of contact with the apical surfaces of opposing anterior lens epithelial cells creates the EFI, a region noteworthy for its high concentration of filamentous actin (F-actin), shown here by labeling with a fluorescent-conjugated phalloidin, which binds specifically to F-actin (Fig. 1A, arrowhead). At E13.5 F-actin was also prominent along lateral borders of neighboring lens epithelial and fiber cells. This pattern of F-actin organization remained a defining feature of the lens throughout development (Fig. 1B,C).

The stability of cadherin junctions is provided through their interaction with cortical F-actin, which is mediated by β-catenin, a molecular regulator that binds directly to the cadherin cytoplasmic domain. At E13.5 β-catenin localizes to lateral borders of lens epithelial cells, at cell-cell interfaces of neighboring primary fiber cells, and in discrete puncta along the newly formed EFI (Fig. 1D). This β-catenin pattern of organization was maintained throughout lens development (Fig. 1D–F). Higher magnification imaging revealed that the β-catenin puncta along the EFI were localized to apicolateral junctions of both lens epithelial and fiber cells (Fig. 1D–F, insets, arrowheads). This result raises interesting questions as to the specific function of opposing apical cadherin junctions at the EFI.

While both E- and N-cadherin link to the cortical actin cytoskeleton through β-catenin, their specific patterns of expression and localization distinguish lens epithelial cells from lens fiber cells. As shown previously, E-cadherin localizes exclusively to lens epithelial cells and is concentrated in junctions along their lateral borders (Fig. 1G–I). E-cadherin junctions were also present as discrete puncta at the apicolateral domains of lens epithelial cells, connecting neighboring epithelial cells near to where they border the EFI (Fig. 1G–I insets, arrowheads). E-cadherin junctions maintain the collective cohesion of epithelia and, in the lens, could give these cells' apical surfaces the adhesive strength required to provide the path along which the apical tips of differentiating secondary fiber cells migrate towards the center of the lens during development.

N-cadherin is expressed by both lens epithelial and fiber cells from early stages of lens development. Our previous studies show that in the epithelial cells of the developing lens N-cadherin junctions are concentrated at these cells' apical domains (Leonard et al., 2011). However, even as early as E13.5, N-cadherin junctions were more prominent along the cell-cell interfaces of primary lens fiber cells then in lens epithelial cells (Fig. 1J). At E14.5, when secondary fiber cells differentiate/elongate from cells in the equatorial epithelium as they turn at the lens fulcrum, E-cadherin junctions are replaced with N-cadherin junctions along their cell-cell borders (Fig. 1K). Interestingly, N-cadherin junctions were enriched specifically in the apical regions of newly differentiating secondary fiber cells, a property maintained throughout lens development (Fig. 1J–L, insets, arrowheads). The adjacent positions of the N-cadherin junctions located in the apical tips of lens epithelial and fiber cells suggested a coordinated function distinct from the N-cadherin junctions that form along the lateral borders of differentiating lens fiber cells. We investigated whether N-cadherin junctions regulate the collective migration of the apical domains of new lens fiber cells along the EFI, contributing to their elongation.

Loss of N-cadherin inhibits migration of fiber cell anterior tips along the EFI leading to aberrant fiber cell elongation

To investigate the function of N-cadherin junctions in the morphogenesis that results in formation of the highly-elongated lens fiber cells during lens development and the generation of the unique lens structure essential to its transparency we created a lens-specific N-cadherin conditional knockout mouse (N-cadcKO). This was accomplished by mating the N-cadherin flox/flox mouse with the MLR10Cre mouse, a transgenic line that expresses Cre under the transcriptional control of the minimal αA-crystallin promoter, modified by the insertion of PAX6 consensus binding sites. Essential to our studies of the role of N-cadherin in fiber cell morphogenesis, this Cre recombinase is not expressed until the lens vesicle stage (E10.5), and then specifically in the posterior lens epithelial cells that become primary lens fiber cells. By E12.5, the MLR10 Cre is expressed throughout the lens. Immunolabeling demonstrated that N-cadherin was effectively deleted from the N-cadcKO lens by E13.5 (Fig. 2A,B). The loss of N-cadherin in the N-cadcKO lens was confirmed by immunoblot analysis at E16.5 (Fig. 2C,D).

Use of the lens-specific MLR10-Cre distinguishes our findings from an earlier study in which LeCre deletes N-cadherin at the placode stage when head ectoderm becomes specified as lens (Pontoriero et al., 2009), as MLR10-Cre N-cadherin is not deleted from the lens until primary fiber cell differentiation. Also, in our N-cadcKO mouse Cre is expressed only in the lens, making it possible to investigate the mechanistic action of N-cadherin in fiber cell differentiation separate from any secondary effects of deleting N-cadherin in other ocular tissues. The effect of N-cadherin loss on lens morphogenetic differentiation was investigated by confocal microscopy imaging of N-cadcKO lens cryosections labeled for F-actin (Fig. 2I–L), a cytoskeletal element previously shown to be assembled along cell borders of lens epithelial and fiber cells, delineating lens cytoarchitecture (Leonard et al., 2011).

At E13.5, the absence of N-cadherin had no overt effects on primary lens fiber cell differentiation. Primary fiber cells elongated coordinately and as a cohesive sheet as they

differentiated, moving directionally in a straight path towards the anterior of the lens (Fig. 2I, arrow). As in wild-type lenses, the apical surfaces of N-cadherin-deficient primary fiber cells interfaced directly with the opposing apical surfaces of the anterior epithelial cells to form the EFI. Significant defects were detected at E13.5 only in the newest, most cortical fiber cells that had begun to form in the region just adjacent to the lens equator. While in normal E13.5 lenses the apical tips of these cortical fiber cells had migrated towards the anterior of the lens (Fig. 2E, arrowhead), in the N-cadcKO lenses at E13.5 the apical tips of these cells did not migrate anteriorly and remained linked to one another along the equatorial plane at their F-actin-rich apical domains (Fig. 2I, arrowhead). These cells are likely to represent the earliest differentiating secondary lens fiber cells that originate in the equatorial epithelium and begin their morphogenetic differentiation as they turn at the lens fulcrum. This hinge region, located along the posterior-most region of EFI, is where the apical surfaces of the newly differentiating fiber cells make their first contact with the apical surfaces of the adjacent equatorial epithelial cells.

In the normal E14.5 lens, the apical tips of these differentiating fiber cells have continued to migrate along the EFI concurrent with the migration of their basal surfaces along the posterior lens capsule. This coordinated migration of the fiber cells' apical and basal surfaces of lens fiber cells creates highly elongated, tightly packed, concave cell structures that envelope the fiber cells that had differentiated before them (Fig. 2F). By E14.5, secondary fiber cells differentiating in the absence of N-cadherin exhibited pronounced elongation defects due to failure of their apical tips to move anteriorly along the apical surfaces of the equatorial epithelium (Fig. 2J, arrowhead). These aberrantly elongating secondary fiber cells have a rich apical cortical actin cytoskeleton, and their apical surfaces remained closely linked and linearly aligned across the lens equator, contiguous with the equatorial epithelium. In contrast, the migration of the basal tips of secondary fiber cells along the posterior lens basement membrane capsule was unaffected by the loss of N-cadherin, as this interaction is mediated by β1 integrin matrix receptors (Menko and Philip, 1995). Primary fiber cells that had initially elongated and contacted the overlying anterior epithelium exhibited early signs of dysmorphogenesis at E14.5, and in some areas these cells had lost their association with the overlying anterior epithelium (Fig. 2J, arrow).

The differentiation of fiber cells in the absence of N-cadherin resulted in even more significant defects at later stages of development. At E16.5 we observed that secondary lens fiber cells had elongated only part of the way towards the lens anterior, their apical tips positioned about half way between the anterior epithelium and the posterior capsule (Fig. 2K, closed arrowhead). These elongation-blunted secondary fiber cells were now extended further across the developing lens. Fiber cells with this altered pattern of morphogenesis continued to increase in number as new secondary fiber cells were added with time from the region of the lens fulcrum, each aligned along the equatorial plane and contiguous with the equatorial epithelium. Interestingly, these lens fiber cells maintained their lateral cell-cell contacts in the absence of N-cadherin junctions (Fig. 2K, arrow). While the differentiating fiber cells in the N-cadcKO lens were greatly shortened, measurements revealed that they had increased greater than 1.5-fold in width, from 0.016μm to 0.026μm (Fig. 2P). Primary fiber cells that had initially elongated normally had become highly disorganized by E16.5, and displayed significant irregularities that appeared as a loss of linearity along their lateral

cell membranes (Fig. 2K, open arrowhead). Similar morphogenetic defects were observed in N-cadcKO lenses at E18.5. Secondary fiber cells continued to be added at the lens fulcrum and were extended as a contiguous sheet across much of the width of the lens, elongating in an anterior direction no farther than the lens equatorial plane (Fig. 2L). The impact of the failure of secondary fiber cells to elongate in the absence of N-cadherin on morphogenesis of the lens was determined by measuring lens width (across the lens equator), height (anterior to posterior), and area (calculated as an ellipse) from E13.5 through E18.5 (Fig. 2M–O). By E18.5 lens area was significantly reduced, primarily due to a decrease in lens height, and consistent with the elongation failure of secondary lens fiber cells. Together, our studies of the N-cadcKO lens revealed that N-cadherin junctions have an essential role in lens morphogenesis by mediating the migration of the apical surfaces of differentiating secondary fiber cells along the EFI.

We also examined the fiber cell morphogenetic defects that occur in N-cadcKO lenses by co-labeling lens sections at E16.5 for F-actin and WGA, a lectin that binds to cell membranes (Fig. 3A–F). In the embryonic lens, WGA binds to membrane-bound organelles and vesicles as well as to the plasma membrane resulting in a diffuse staining pattern that provides a distinct view of the organizational state of new fiber cells from that of F-actin labeling. WGA-labeling confirmed the altered morphogenesis of primary and secondary fiber cells in the N-cadcKO lens (Fig. 3D,F) that was shown by labeling for the F-actin cytoskeleton (Fig. 3E,F). These results also showed that there was a more intense WGA labeling of elongation-defective secondary fiber cells than of primary lens fiber cells (Fig. 3D), revealing another distinction between these two compartments of the N-cadcKO lens at E16.5. The WGA labeling intensity of secondary fiber cells that failed to elongate in the N-cadcKO lens was like that of the youngest secondary fiber cells located just past the lens fulcrum in normal lenses (Fig. 3A,C). This higher-level WGA staining is consistent with a higher content of membrane-bound organelles and vesicles in lens fiber cells prior to when they begin to lose organelles during formation of the lens Organelle Free Zone (OFZ).

Dysmorphogenesis of N-cadcKO lens fiber cells does not induce apoptosis

In the N-cadcKO lens primary lens fiber cells exhibited significant disorganization as early as E14.5. We investigated whether this reflected a degenerative process associated with their apoptotic cell death by performing TUNEL assay analysis (Fig. 4). No TUNEL positive nuclei were detected in N-cadcKO lenses at E16.5 (Fig. 4B,F), a full two days after primary fiber cells that differentiated in the absence of N-cadherin first exhibited dysmorphogenesis (see Fig. 2J, E14.5). Primary fiber cells of N-cadcKO lenses remained viable throughout most of lens development, and the dysgenesis of primary fiber cells that occurs in the absence of N-cadherin did not lead to their degeneration and apoptotic cell death until the latest stage of development, E18.5 (Fig. 4D). In wild type lenses, a large OFZ has formed by E18.5 (Fig. 4C,G). In E18.5 N-cadcKO lenses the OFZ was often smaller than in normal lenses and flanked on either side by a few layers of fiber cells with pyknotic, TUNEL positive nuclei that most likely represent the ongoing expansion of the OFZ (Fig. 4D,H). This finding suggested that there is a developmental delay in the formation of the OFZ in the absence of N-cadherin. Note that no TUNEL labeling was detected in the elongation-defective secondary fiber cells of the N-cadcKO lenses at any stage of development. Their

nuclei formed a second nuclear region that was linearly aligned below the equatorial plane. The distribution of these nuclei emphasized the continuity between the aberrantly elongating fiber cells and the equatorial epithelial cells of the N-cadcKO lenses. The retention of nuclei by the secondary fiber cells throughout lens development suggests that their defective morphogenesis has consequences on other aspects of lens development.

Loss of N-cadherin compromises secondary lens fiber cell elongation without blocking differentiation

The fiber cell elongation defect in N-cadcKO lenses suggested that the role of N-cadherin in lens development might be primarily morphogenetic and not impact expression of lens fiber cell differentiation-specific proteins. To investigate this possibility, E16.5 N-cadcKO lens sections were immunolabeled for βB-crystallin (Fig. 5A,C,D,F), a cytoplasmic protein with a fiber cell-specific pattern of expression. βB-crystallin is a determinant of lens transparency and refractive function. Confocal image analysis showed that expression of βB-crystallin by lens fiber cells was unaffected when these cells differentiated in the absence of N-cadherin (Fig. 5D,F). In the N-cadcKO lens, βB-crystallin was expressed throughout the fiber cell compartment, including the elongation-defective secondary fiber cells from the time they emerged from the posterior lens equatorial epithelium at the lens fulcrum. In addition, there was no change in βB-crystallin's pattern of localization. This finding was consistent with a previous study showing expression of βB-crystallin by lens fiber cells when N-cadherin was knocked out earlier, at the lens placode stage (Pontoriero et al., 2009). We also performed immunoblot analysis to quantify and compare βB-crystallin expression between N-cadcKO and normal lenses at E16.5. The results confirmed that βB-crystallin expression was maintained in the absence of N-cadherin (Fig. 5G,H), and provided further evidence that the morphogenetic defects of secondary fiber cells in the N-cadcKO lens did not affect their ability to express a key differentiation-specific protein.

Atypical maintenance of E-cadherin in new fiber cells of the N-cadcKO lens does not rescue their migration defect

E-cadherin is a defining feature of lens epithelial cells and is normally turned off as lens fiber cells begin to differentiate (Takeichi et al., 1986; Takeichi, 1988; Choi et al., 2007; Pontoriero et al., 2009; Audette et al., 2016). Here we show the exclusive expression of E-cadherin by the epithelial cells of the wild type lens at E16.5 (Fig. 6A,C), and that the loss of N-cadherin did not affect E-cadherin's localization in lens epithelial cells (Fig. 6D,F, arrow). Surprisingly though, in the N-cadcKO lens E-cadherin was retained by the differentiating secondary lens fiber cells positioned just past the lens fulcrum (Fig. 6D,F,G, arrowhead). This aberrant expression of E-cadherin in lens fiber cells was primarily a feature of their apicolateral domains. Co-immunolabeling N-cadcKO lens sections for both E-cadherin and βB-crystallin showed that the E-cadherin-retaining secondary fiber cells expressed βB-crystallin and confirmed that E-cadherin was present in cells that had initiated their differentiation (Fig. 5K,L). The persistence of E-cadherin at the apicolateral borders of βB-crystallin-expressing, N-cadherin-deficient, differentiating lens fiber cells demonstrated that it is not necessary to suppress E-cadherin for lens cells to express a differentiation-specific protein profile. Although E-cadherin was retained at the apicolateral domains of this subpopulation of fiber cells in N-cadcKO lenses, it was not able to replace N-cadherin

function and rescue their elongation/migration defect. β-catenin, which binds to the cytoplasmic domain of classical cadherins and mediates the formation of cadherin junctions, had a similar distribution to E-cadherin in N-cadcKO lenses, including at the apicolateral junctions of new lens fiber cells (Fig. 6K,N, closed arrowheads). However, β-catenin is lost along with N-cadherin in all but the E-cadherin-retaining lens fiber cells (Fig. 6K, open arrowhead). This result is consistent with our previous findings that N-cadherin is a principal cadherin of differentiating lens fiber cells (Leonard et al., 2011; Leonard et al., 2013), and suggests that no other classical cadherin replaces N-cadherin in the differentiating lens fiber cells of the N-cadcKO lens.

ZO-1/F-actin junctions link apicolateral domains of dysmorphogenic secondary fiber cells in N-cadcKO lenses

The apical surfaces of the elongation-defective secondary fiber cells in N-cadcKO lenses were defined by a prominent cortical actin cytoskeleton (Fig. 6G,N, arrows). These apical actin structures extended unbroken across the cells' anterior-most surfaces, linking the secondary fibers into a contiguous sheet. We examined what junctional elements were responsible for linking the cortical actin cytoskeleton across the apical domains of the secondary fiber cells in the N-cadcKO lens. Co-labeling of F-actin with both E-cadherin and β-catenin showed that E-cadherin/β-catenin junctions only partially overlapped with the F-actin apical border, with much of the apical actin cytoskeleton concentrated anterior to the E-cadherin/β-catenin junctions (Fig. 6G,N, arrow). In addition, cortical actin structures extended along the apical surfaces of the secondary lens fiber cells significantly beyond the subpopulation that atypically expressed apicolateral E-cadherin/β-catenin junctions (Fig. 6G,N, arrows).

We next examined whether ZO-1, a cell-cell junctional molecule capable of maintaining the contiguity of a group of morphogenetically-coordinated cells, mediates the adhesion of the apical surfaces of neighboring elongation-defective secondary lens fiber cells. This cell-cell adhesion protein connects to the F-actin cytoskeleton and, in normal lenses, was localized to the EFI, as reported previously (Fig. 6O,Q arrows, and Wang et al., 2016). Confocal image analysis of N-cadcKO lens sections showed that ZO-1 was highly localized to the apical-most domains of the elongation-defective secondary fiber cells (Fig. 6R,U, arrow) and coincident with the apical F-actin border (Fig. 6T, and U, arrow). ZO-1 junctions extended linearly across the apical domains of the secondary fiber cells, and was a prominent feature of this cell compartment. The most centrally located of the migration-defective secondary fiber cells begin to lose apicolateral cohesiveness coincident with disruption of the ZO-1/F-actin-rich apical border (Fig. 6R,S,T, arrowhead). These results suggest that ZO-1 junctions are responsible for maintaining the collectivity of secondary fiber cells in N-cadcKO lenses.

Potential role for lens channel proteins in maintaining lateral cell adhesion in the absence of N-cadherin

In the normal lens, N-cadherin forms adherens junctions that link neighboring fiber cells along their cell-cell borders (Cheng et al., 2016). Therefore, it was somewhat surprising that the loss of N-cadherin did not have a significant effect on the ability of differentiating secondary lens fiber cells to form lateral adhesive interactions. While best-known for their

role as lens channel/communication proteins, Connexin 50 (Cx50) and Aquaporin 0 (Aqp0) also can have cell adhesive function (Nielsen et al., 2001; Nielsen et al., 2003; Chung et al., 2007; Kumari and Varadaraj, 2009; Liu et al., 2011; Lo et al., 2014; Wang et al., 2016). Here, we investigated Cx50 and Aqp0, both molecules with fiber cell-specific patterns of expression in the lens, as potential mediators of lateral adhesive contacts between these cells in the N-cadcKO lens. Immunoblot and immunofluorescence studies showed that the absence of N-cadherin during lens fiber cell differentiation resulted in a lower expression of Cx50 protein (Fig. 7G,H), and altered Cx50 distribution (Fig. 7D,F). The distribution of this protein appeared more punctate and discontinuous along fiber cell lateral interfaces (Fig. 7D, arrow) then in normal fiber cells (Fig. 7A). Also, elevated levels of Cx50 were observed in the subpopulation of migration-defective lens fiber cells that are located just past the lens fulcrum, especially in their more anterior aspects (Fig. 7D, arrowhead). These altered patterns of Cx50 localization suggest that N-cadherin may play a role in organizing Cx50 junctions in the membrane of normal lens fiber cells, and that their function might be impacted in the absence of N-cadherin junctions.

In contrast to the Cx50 results, confocal analysis of sections from N-cadcKO lenses immunolabeled for Aqp0 showed a greatly increased localization of this differentiation-specific lens protein along the cell-cell borders of secondary lens fiber cells differentiating in the absence of N-cadherin (Fig. 7L, arrow), compared to wildtype (Fig. 7I,K, higher intensity image shown as inset in I). However, immunoblot analysis showed that expression of Aqp0 protein in the N-cadcKO lens remained unchanged (Fig 7O,P). The increased immunolocalization of Aqp0 at lateral cell interfaces of fiber cells in the N-cadcKO lens with no change in protein level may reflect a disruption of membrane protein scaffolding and Aqp0 interacting-proteins leading to an altered functional organization of Aqp0 and greater antibody access. Immunolabeling for Aqp0 also provided further insight into the disorganization of the primary fiber cell compartment that occurs in the absence of N-cadherin by E16.5 (Fig. 7L, arrowhead). This disorganization and loss of linearity of the lateral contacts between primary lens fiber cells suggested that N-cadherin may play a role in the alignment of fiber cell lateral borders.

Fiber cell migration defect of the N-cadcKO lens may be linked to disregulation of EphA2 in the absence of N-cadherin junctions

During tissue development Eph/ephrin signaling regulates cell guidance processes and sets up tissue boundaries (Theveneau et al., 2010; Theveneau and Mayor, 2012; Gartner et al., 2015; Roycroft and Mayor, 2016). Importantly, N-cadherin labeling is disrupted and sometimes absent in EphA2−/− lens fiber cells (Cheng and Gong, 2011; Cheng et al., 2013). Like our findings with the N-cadcKO mouse, lenses of EphA2−/− knockout mice retain expression of E-cadherin in newly differentiating cortical lens fiber cells (Cheng and Gong, 2011; Cheng et al., 2013). The persistence of E-cadherin in fiber cells in the absence of EphA2 may be directly related to their loss of N-cadherin junctions. The defects in N-cadherin junctions of EphA2−/− lenses, the maintenance of E-cadherin junctions, and the disorganization of the lens fulcrum region where the transition from epithelial to fiber cells occurs suggests that EphA2 may function in stabilizing N-cadherin junctions. Therefore, we investigated whether the morphogenetic defects in fiber cell elongation in the N-cadcKO

lenses might involve the dysregulation of EphA2. Immunofluorescence (Fig. 8D,F) and immunoblot (Fig. 8G,H) analysis of E16.5 N-cadcKO lenses revealed that in the absence of N-cadherin the expression of EphA2 was significantly increased. The immunolocalization studies showed that EphA2 was highly localized to cell-cell borders of both primary and secondary lens fiber cells of N-cadcKO lenses (Fig. 8D,F). This elevated expression of EphA2 in the absence of N-cadherin suggests that there may be a coordinate regulatory loop between these two molecules in differentiating lens fiber cells, and the enhanced EphA2 at cell-cell interfaces may reflect a role in maintaining cell-cell adhesion. However, the increase in EphA2 at fiber cell borders in the absence of N-cadherin was not able to rescue the inability of newly differentiating secondary fiber cells to migrate along the EFI.

Dysregulation of signaling pathways associated with cell migration in lens cells differentiating in the absence of N-cadherin

The loss of N-cadherin prior to secondary fiber cell differentiation impairs fiber cell elongation primarily by inhibiting the migration of the apical domains of these differentiating fiber cells along their normal path across the anterior surfaces of the overlying lens epithelium. This anterior migration defect occurred without any observable impact on the migration of these cells' basal surfaces along the posterior lens capsule. Since myosin II and Rac have crucial roles in regulating cell migration, we investigated whether their localization or activation state was impacted by the absence of N-cadherin in differentiating lens fiber cells. The impact of the loss of N-cadherin on the expression and activation state of myosin II in differentiating lens fiber cells was investigated with both immunolocalization and immunoblot approaches (Fig. 9). While we observed no significant change in the localization of myosin II in the absence of N-cadherin (Fig. 9E), phospho-myosin, the activated form of this protein, was increased along cell-cell interfaces of lens fiber cells (Fig. 9F,L, arrows). Elevated myosin activation was greatest in secondary fiber cells, and was especially pronounced at late stages of lens development (E18.5) (Fig. 9L). Fiber-cell specific increase in p-myosin was confirmed by analysis of pixel intensity in the epithelium and fiber cells of E16.5 and E18.5 lenses (Fig 9I,N). Immunoblot analysis showed that both total myosin II and phospho-myosin protein levels were increased in the absence of N-cadherin, with a slightly greater increase in phospho-myosin than myosin II (Fig. 9O,P), suggesting that the phenotype observed could result from elevation of both an increase in myosin protein expression and its activation. The increased activation of myosin II in N-cadcKO lenses was correlated with the induction of myosin light chain kinase (MLCK), which regulates myosin II activity through its phosphorylation of myosin II light chain (Fig. 9H). The increased activation of myosin along lateral interfaces of differentiating lens fiber cells in the absence of N-cadherin could negatively impact fiber cell elongation by inducing excessive actomyosin contraction.

The RhoGTPase Rac is a core element of pathways regulating cell migration (Maddala et al., 2011, Ridley, 2015). Here, we investigated whether there was region-specific inhibition of the activation of Rac in fiber cells differentiating in the absence of N-cadherin, consistent with the anterior migration defect of fiber cells in N-cadcKO lenses. We found that Rac1 was widely distributed and activated in the normal E16.5 embryonic lens, where it was most highly localized to cell-cell interfaces of the newly differentiating fiber cells located just past

the lens fulcrum (Fig. 10A, arrow), and in discrete puncta along the basement membrane where the basal surfaces of lens cells contact the lens capsule (Fig. 10A,C, arrowheads). It was previously shown that lens-specific deletion of Rac1 causes extension defects at both the apical and basal surfaces of differentiating fiber cells (Maddala et al., 2011). In N-cadcKO lenses at E16.5, much of the lens fiber cell region displayed a significant loss of cytoplasmic Rac1 (Fig. 10B), which was confirmed by immunoblot analysis (Fig. 10E,F). In contrast, the newly differentiating secondary fiber cells localized just adjacent to the lens fulcrum that failed to migrate along the EFI retained a high level of Rac1 at their cell borders in the absence of N-cadherin (Fig. 10B, arrowhead). Rac1 activation (Rac1-GTP) was suppressed in all regions normally rich in N-cadherin junctions, including the newly differentiating fiber cells that retained a high level of Rac1 expression in the absence of N-cadherin (Fig. 10D). That the loss of N-cadherin blocked Rac activation without affecting Rac expression in the population of newly differentiating lens fiber cells that fail to migrate along the EFI (Fig. 10B,D, arrowheads) suggests that it is the loss of Rac activity, not Rac protein, in the N-cadcKO lens that is associated with the lens fiber cell migration defect. Rac activity remained high at the basal surfaces of lens epithelial and fiber cells of the N-cadcKO lens (Fig. 10D, arrow), where Rac would be activated downstream of integrin receptors that engage the lens basement membrane, and not by N-cadherin. This analysis of Rac shows for the first time that there is a direct link between N-cadherin function and Rac activation in the newly elongating cells of the developing lens, and suggests that the loss of N-cadherin-dependent Rac activity could be responsible for the failure of these newly differentiating lens fiber cells to migrate along the EFI.

DISCUSSION

N-cadherin is a versatile member of the cadherin receptor family, with functions that include a unique role in the regulation of cell migration (Theveneau et al., 2010; Theveneau and Mayor, 2011; Menko et al., 2014; Priya and Yap, 2015; Audette et al., 2016). In this role, N-cadherin has been implicated in various developmental processes including the collective movement of neural crest cells (Theveneau and Mayor, 2011; Theveneau and Mayor, 2012; Barriga and Mayor, 2015; Kalcheim, 2015; Scarpa et al., 2015). N-cadherin is expressed by all cells of the developing lens. In the undifferentiated lens epithelium N-cadherin is most highly concentrated along their apicolateral domains (Bassnett et al., 1999; Ferreira-Cornwell et al., 2000; Leong et al., 2000; Menko, 2002), and in differentiating lens fiber cells N-cadherin localizes to all regions of cell-cell contact, both lateral and apical (Leonard et al., 2011). Although N-cadherin junctions are often targeted as a consequence of other molecular knockouts that cause lens dysmorphogenesis (Cooper et al., 2008; Cheng and Gong, 2011; Son et al., 2012; Cheng et al., 2013; Biswas et al., 2016), the mechanism of action by which N-cadherin functions in lens fiber cell differentiation had remained elusive. Studies with our N-cadherin lens conditional knockout (N-cadcKO), in which N-cadherin is eliminated at the time of lens fiber cell differentiation, have now revealed that N-cadherin junctions mediate the migration of the apical tips of differentiating secondary lens fiber cells along the apical surfaces of the epithelium, which is necessary for fiber cell elongation and lens morphogenesis.

Interestingly, the loss of N-cadherin did not effect the elongation of the primary fiber cells that extend as a coordinated sheet of cells in a direct path from the posterior of the lens vesicle until they reach the anterior epithelium. The elongation of secondary fiber cells as they emerge just past the lens fulcrum involves a morphogenetic process quite distinct from that of primary fiber cells that is dependent on N-cadherin junctions. In the absence of N-cadherin, secondary lens fiber cells fail to elongate beyond the equatorial plane due to the failure of their apical surfaces to migrate along the EFI, but maintain apical collectivity through ZO-1 junctional linkages. Studies with the N-cadcKO lenses have revealed some unexpected new insights into the morphogenetic processes of development. The suppression of E-cadherin junctions that are normally expressed by epithelial cells is believed to be essential to executing the differentiation program in tissues like the lens. However, we now show that in the absence of N-cadherin, newly differentiating secondary lens fiber cells initially maintain their apicolateral E-cadherin junctions. While this aberrant expression of E-cadherin was not able to rescue the migration defect in N-cadcKO lenses that prevents secondary fiber cell elongation, the presence of E-cadherin junctions did not interfere with fiber cell differentiation state-specific gene expression. Furthermore, the continued expression of lens fiber cell-specific proteins in N-cadcKO lenses revealed that lens differentiation-specific gene expression (Hoang et al., 2014; Audette et al., 2016) is signaled independently of lens fiber cell morphogenesis. It is also notable that the loss of N-cadherin, which in wildtype lenses is enriched both in and at the base of interlocking protrusions of lateral fiber cell membranes (Biswas et al., 2016; Cheng et al., 2016), results in increased immunolabeling for Aqp0 in the absence of enhanced Aqp0 expression. Since Aqp0 also localizes to fiber cell protrusions (Lo et al., 2014), this result suggests that N-cadherin may play a role in the functional organization of Aqp0 in the lateral membranes of differentiating lens fiber cells. In addition, the developmental delay in formation of the OFZ in the N-cadcKO lens and the failure of the migration-defective secondary fiber cells to undergo denucleation at the time of OFZ formation suggests that signals regulating nuclear degradation, such as inactivation of JNK (Basu et al., 2014) and/or Cdk1 activation (Rowan et al., 2016), rely on proper lens fiber cell morphogenesis and possibly N-cadherin-dependent cell-signaling pathways.

Our findings that EphA2 is significantly upregulated in the N-cadcKO lens support the conclusion that there is a close relationship between N-cadherin and EphA2 function in the developing lens. Importantly, N-cadherin junctions are disrupted and sometimes lost in lenses of the EphA2−/− mouse, and like in the N-cadcKO lens, E-cadherin persists into the fiber cell zone (Cheng and Gong, 2011; Cheng et al., 2013). The disorganization of lens cells as they begin to differentiate in the absence of EphA2−/− suggests that this outcome may be connected to the loss of N-cadherin junctions in these cells, and that EphA2 may function directly in stabilizing these junctions. These results, together with our findings that EphA2 expression is dysregulated in the absence of N-cadherin, suggest closely coordinated functions of EphaA2 and N-cadherin for the proper morphogenetic differentiation of the lens.

N-cadherin junctions impact actin cytoskeletal dynamics through downtream signaling regulators, and we previously show that N-cadherin junctions serve as epicenters for actin regulation in differentiating lens fiber cells (Leonard et al., 2011). Myosin II is a molecular

motor that, through its interaction with F-actin, underlies cell contractility and movement (Rao and Maddala, 2006; Maddala et al., 2007; Doyle et al., 2015; Aranjuez et al., 2016; Han and de Rooij, 2016; Mui et al., 2016), and also has an important role in minimizing curvature of cell-surface microdomains (Elliott et al., 2015). Myosin activity has an important role in maintaining lens function as exposure to an MLCK inhibitor induces opacities in lens organ cultures (Maddala et al., 2007). Our studies provide evidence that N-cadherin junctions regulate the level of myosin activation. In the N-cadcKO lens myosin activity increased specifically along the lateral borders of differentiating secondary fiber cells. This enhanced myosin activity could lead to an increase in actomyosin contraction and contribute to the elongation defect of lens fiber cells. Since enhanced myosin activation can prevent cell curvature, it is also likely that the increase in myosin activation along cell-cell interfaces of lens fiber cells is responsible for the lack of curvature that is characteristic of the shortened secondary fiber cells of N-cadcKO lenses. This outcome would place N-cadherin in an important mechanistic role of regulating myosin activity to allow differentiating lens fiber cells to curve inwardly as they surround the cells that have differentiated before them.

No significant change in myosin activity was detected at the apical tips of differentiating fiber cells in the N-cadcKO lens, whose interaction with and movement along the overlying epithelium is essential to fiber cell elongation. Therefore, we investigated what other N-cadherin-dependent signaling mechanism might regulate this process. Our results pointed to the regulation of Rac activation in the process of Contact Inhibition of Locomotion (CIL). CIL is best known for its role in the directional, collective migration of the neural crest (Theveneau and Mayor, 2011; Theveneau and Mayor, 2012; Scarpa et al., 2015; Roycroft and Mayor, 2016). In CIL, N-cadherin-mediated cell-cell contacts start a process whereby the RhoGTPase Rac1, which signals assembly of actin at the leading edge of migrating cells, is suppressed at N-cadherin cell-cell contact sites and concurrently activated in an area of the cell that becomes the new leading edge, thereby redirecting the migration path (Theveneau et al., 2010; Scarpa et al., 2015; Roycroft and Mayor, 2016). In CIL, N-cadherin regulation of the activation of Rac1 determines where the actin-dependent cell protrusions necessary for directional migration are formed in the cell (Scarpa et al., 2015). Therefore, N-cadherin cell-cell contact changes the subcellular domain where Rac is activated from the site of initial N-cadherin contact to the lamellipodial edge that will redirect the path of cell migration. This mechanism allows a group of connected cells to move continually and collectively towards a destination required for development, as for fiber cells in the developing lens.

Previous studies show that Rac1 localizes to basal cell surfaces of lens epithelial and fiber cells and along the EFI, and that lens conditional deletion of Rac1 prevents elongation of lens fiber cells along the both the posterior lens capsule and the EFI (Maddala et al., 2011). We now show that N-cadherin function is linked to activation of Rac in the developing lens. Our findings of the dependence of Rac activity on N-cadherin junctions are consistent with a central role for N-cadherin-linked Rac signaling in the movement of fiber cell apical domains along the EFI, as occurs in CIL. Therefore, CIL is a strong candidate for the process that regulates how newly differentiating lens fiber cells are signaled to move along

the EFI after their apical tips make initial contact with the apical domains of opposing lens epithelial cells.

Conclusion

We demonstrate the crucial role of N-cadherin in lens fiber cell morphogenesis and show that E-cadherin is not able to replace N-cadherin function in migration/elongation of newly differentiating fiber cells along the EFI. Our findings with the N-cadcKO lens provide the first evidence that the lens fiber cell elongation process requires N-cadherin-dependent regulation of Rac and myosin, likely directing the migration of fiber cell apical domains along the EFI and curvature of the fiber cells as they differentiate. These discoveries have wide-ranging implications regarding the role of N-cadherin in tissue morphogenesis and regeneration.

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Highlights

- N-cadherin lens-specific conditional knockout prevents lens fiber cell elongation
- N-cadherin regulates migration of fiber cell apical tips along the epithelium
- N-cadherin and EphA2 likely have coordinate functions in lens fiber cell elongation
- Role of N-cadherin in elongation involves regulation of Rac1 and myosin activation
- Loss of N-cadherin does not impact expression of differentiation-specific proteins

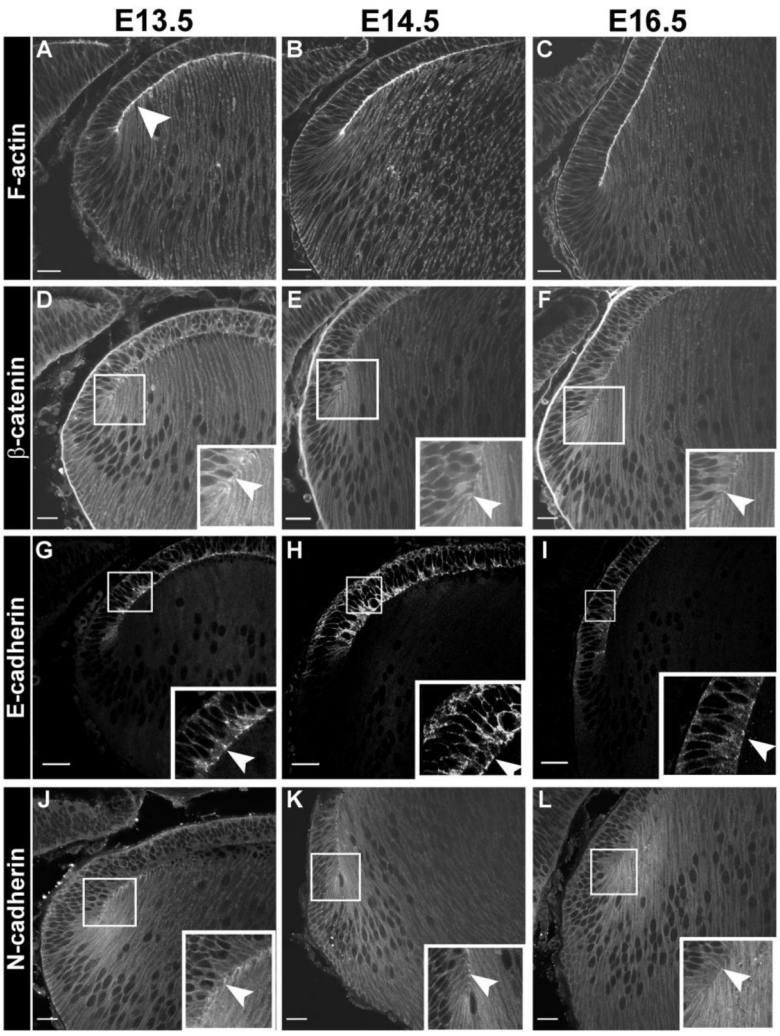

Figure 1. Expression of cadherin junctional proteins and F-actin in the developing lens

Cryosections of E13.5 (A,D,G,J), E14.5 (B,E,H,K), and E16.5 (C,F,I,L) eyes were labeled for F-actin (A,B,C), β-catenin (D,E,F), E-cadherin (G,H,I) or N-cadherin (J,K,L). (A–C) F-actin localized to cell-cell borders and along the epithelial fiber interface (EFI) where epithelial and fiber cell apical tips interact (A, arrowhead). (D–F) β-catenin was localized to cell-cell borders of lens epithelial and fiber cells, and in a punctate pattern along the EFI that is shown as a higher magnification of the boxed areas in insets (arrowheads). (G,H,I) E-cadherin was expressed only in the lens epithelium, including distinct puncta just adjacent to the EFI, shown at a higher magnification of the boxed areas in the insets (arrowheads). (J,K,L) N-cadherin was localized along cell-cell borders of lens epithelial and fiber cells and in a punctate pattern along the EFI shown at a higher magnification of the boxed areas in the insets (arrowheads). (Mag bar=20μm; n=5)

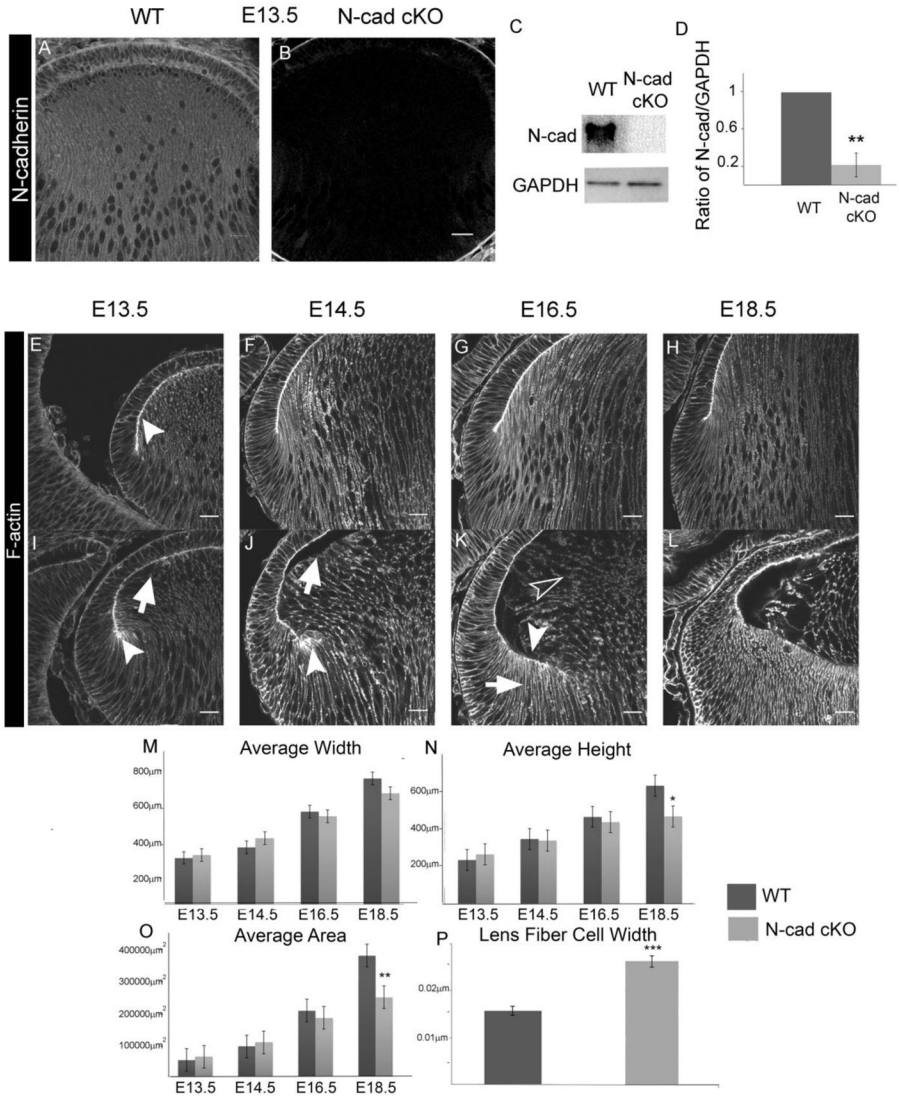

Figure 2. Loss of N-cadherin expression in N-cadcKO lenses blocks migration/elongation of the apical tips of lens fiber cells along the EFI resulting in disrupted lens morphogenesis

Cryosections of E13.5 wildtype (A) and N-cadcKO (B) eyes labeled for N-cadherin show loss of N-cadherin in N-cadcKO lenses by E13.5. (C) Immunoblot for N-cadherin in E16.5 wildtype and N-cadcKO lenses confirms the loss of N-cadherin protein in the N-cadcKO lens, with GAPDH as a loading control. (D) Quantification of immunoblot analysis shows highly significant loss of N-cadherin in knockout lenses, data presented as a ratio to GAPDH. Cryosections of wildtype (E–H) and N-cadcKO (I–L) eyes were labeled for F-actin at E13.5 (E,I), E14.5 (F,J), E16.5 (G,K) and E18.5 (H,L). By E13.5 primary fiber cells of wildtype lenses have elongated coordinately in a straight path from the posterior of the lens to the anterior epithelium (E). A small number of secondary fiber cells have begun to form from the equatorial epithelium, and have migrated anteriorly along the EFI towards the anterior of the lens (E, arrowhead). In N-cadcKO lenses at E13.5 the primary fiber cells have elongated normally, their apical tips interacting with the overlying anterior epithelium (I, arrow), but the apical tips of secondary fibers have failed to migrate along the EFI and

remain clustered adjacent to the lens fulcrum (I, arrowhead). By E14.5 there is increased clustering of secondary fiber cell apical tips at the fulcrum of N-cadcKO lenses (J, arrowhead) due to failure to migrate along the EFI. Primary fibers in these lenses begin to exhibit signs of disorganization and separation from the EFI (J, arrow). Later in development at E16.5, the migration failure of secondary fibers cells differentiating in the absence of N-cadherin causes major morphogenetic defects (K). Secondary fiber cells accumulate, contiguous with the equatorial epithelium but failing to elongate beyond the equatorial plane (K, closed arrowhead). Note that the elongation defective differentiating secondary fiber cells maintain lateral cohesion in the absence of N-cadherin (K, arrow). At this time of development, primary fiber cells become extensively disorganized (K, open arrowhead). The phenotype at E18.5 is similar to E16.5 with continued addition of new secondary fiber cells at the equatorial plane and increased disorganization of lens primary fibers (L). Measurements were made from confocal microscopy images acquired of F-actin-labeled cryosections from E13.5, E14.5, E16.5, and E18.5 wildtype (WT) and N-cadcKO eyes of lens width (M), height (N), and area (O). By E18.5 there was significant loss of height leading to decreased area in N-cadcKO lenses. (P) Shortened fiber cells that failed to elongate along the EFI in N-cadcKO lenses at E16.5 had a corresponding increase in width. (Mag bar=20μm; A–D n=12; E–L n=12; $p<0.05$*;$p<0.01$**; $p<0.001$***).

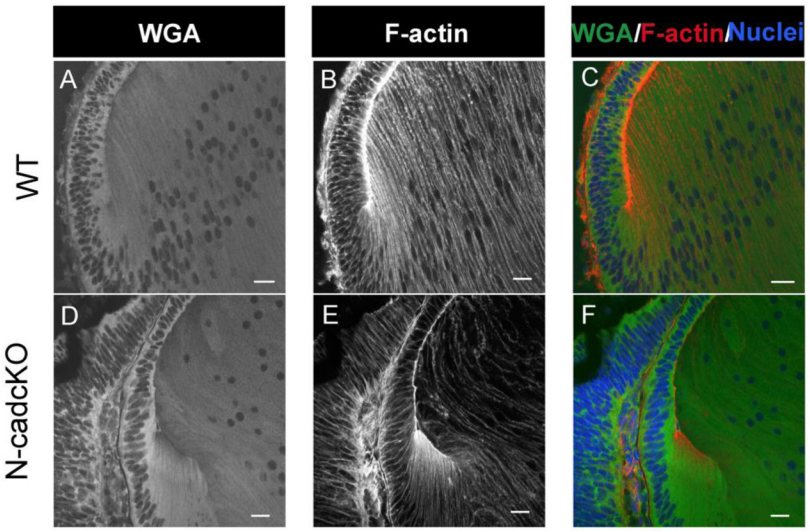

Figure 3. Absence of N-cadherin alters lens fiber cell morphology and membrane distribution

(A–F) Cryosections at E16.5 from wildtype (A–C) and N-cadcKO (D–F) eyes were labeled with F-actin (B,C,E,F) and WGA (A,C,D,F) to examine lens morphology. N-cadcKO lenses showed a differential labeling of WGA between primary fiber cells and secondary fiber cells (D,F), highlighting the distinction between these compartments in the N-cadcKO lens and the dysmorphogenesis of differentiating secondary fiber cells seen also with F-actin labeling (E,F). (Mag bar=20μm; n=3)

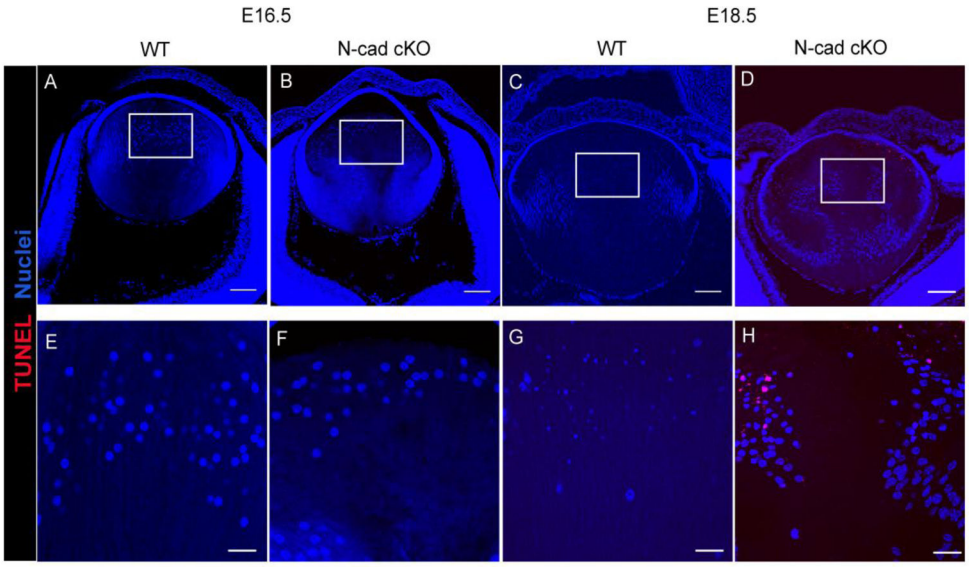

Figure 4. Absence of N-cadherin alters lens fiber cell nuclear distribution but does not result in cell death

Cryosections at E16.5 (A,B,E,F) and E18.5 (C,D,G,H) from wildtype (A,C,E,G) and N-cadcKO (B,D,F,H) eyes were labeled by TUNEL assay (red) and counterstained for nuclei (blue). Boxes in A–D represent areas shown in E–H, respectively. Neither wildtype nor N-cadcKO lenses demonstrated TUNEL positivity at E16.5 (A,B,E,F). By E18.5 wildtype lenses (C,G) had formed a large Organelle Free Zone (OFZ) in the center of the lens, as is typical of this stage of development. N-cadcKO lenses (D,H) had formed a smaller OFZ at E18.5, which was flanked on either side by a few layers of fiber cells with TUNEL-positive nuclei, likely representing the ongoing expansion of the OFZ in the mutant lenses. Cells in the primary fiber zone began to exhibit TUNEL-positive labeling at E18.5 (D) (mag bar in low power images= 200μm; in high power images=20μm; n=8)

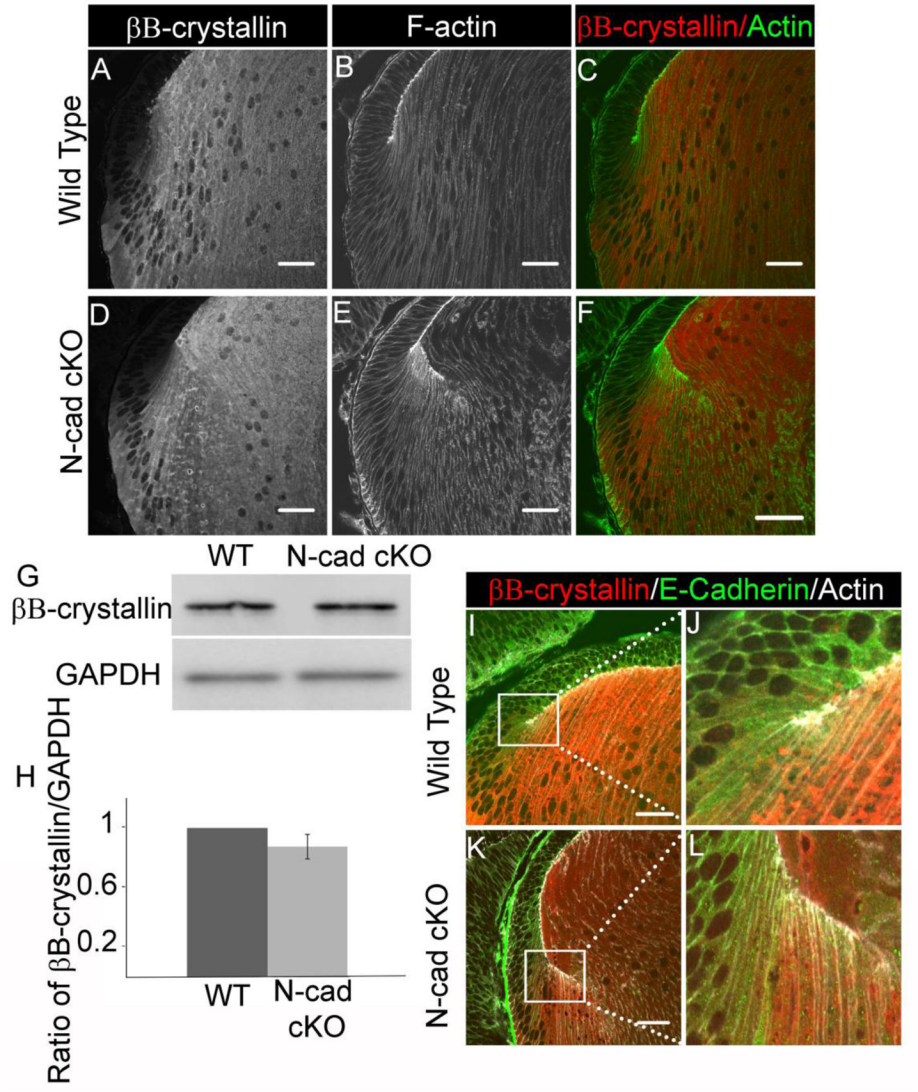

Figure 5. βB–crystallin is expressed in N-cadcKO lenses, including newly differentiating fiber cells that aberrantly express E-cadherin

Cryosections of E16.5 wild-type (A,B,C,I,J) and N-cadcKO (D,E,F,K,L) eyes were labeled for (A,C,D,F,I–L) βB–crystallin, (I–L) E-cadherin, and (B,C,E,F,I–L) F-actin. βB–crystallin expression was maintained in lens fiber cells in the absence of N-cadcKO (D,F). In the N-cadcKO lens E-cadherin expression fails to be turned off in new secondary fiber cells located near the lens fulcrum whose differentiation is confirmed by their expression of βB–crystallin (K,L). βB–crystallin expression was also examined by immunoblot analysis of proteins from E16.5 wild-type and N-cadcKO lenses, which confirmed that there was no significant change in βB–crystallin protein level (G,H). (mag bar=20μm; n=3; $p<0.05$*;$p<0.01$**; $p<0.001$***)

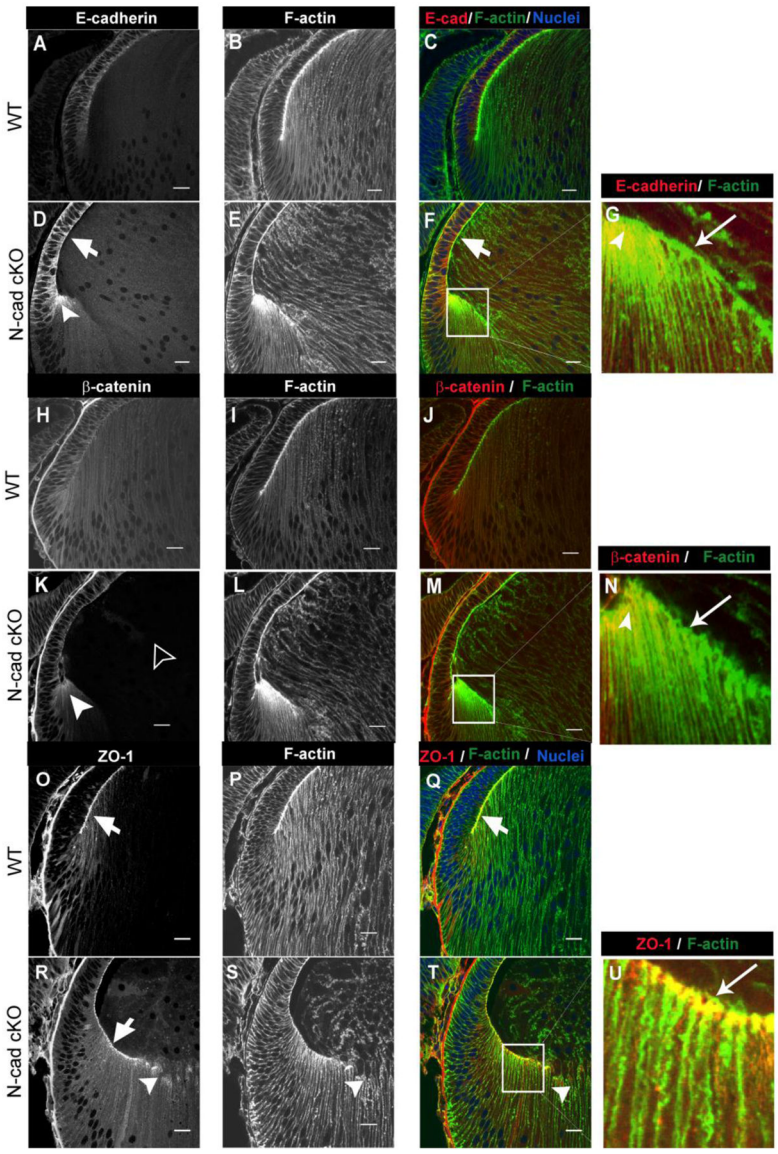

Figure 6. E-cadherin/β-catenin junctions maintained but apical ZO-1 junctions more extensive, linking apical domains of migration-defective fiber cells in the N-cadcKO lens

Cryosections of wild-type (A–C,H–J, O–Q) or N-cadcKO (D–G,K–N,R–U) eyes were labeled for E-cadherin (A,C,D,F,G), β-catenin(H,J,K,M,N), ZO-1(O,Q,R,T,U), F-actin (B,C,E–G,I,J,L–N,P,Q,S–U), and nuclei (C,F,Q,T) at E16.5. E-cadherin is restricted to the epithelium of normal lenses (A,C). In N-cadcKO lenses E-cadherin is maintained in lens epithelial cells (arrow) and expressed aberrantly in the apical tips of migration-defective, differentiating cortical fiber cells close to the lens equator (D,G, arrowhead; G is a high magnification of the boxed area in F). β-catenin, which normally localizes to all cell-cell borders (H,J), is retained only by cells expressing E-cadherin junctions in N-cadcKO lenses (K,M), including the apical tips of the migration-defective cortical fiber cells near the lens fulcrum (K,N, closed arrowhead; N is a high magnification of the boxed area in M. No β-catenin labeling was detected in the remainder of the lens fiber cell mass (K, open

arrowhead). ZO-1 is most highly expressed at the EFI of wildtype lenses (O,Q, arrows). In the N-cadcKO lens, ZO-1 junctions were maintained along the apical domains of lens epithelial cells and extended across the apical domains of the secondary fiber cell population that fails to elongate and migrate along the EFI (R,T,U, denoted by arrows in R and U; U is a high magnification image of the area boxed in T), co-localized with F-actin (T,U). Loss of ZO-1 correlates with discontinuity of the fiber cells' apical domains (R,S,T, arrowhead). High magnification images of E-cadherin (G, arrowhead) and ZO-1 (U, arrow) co-labeled for F-actin show ZO-1 is localized apical to E-cadherin and β-catenin and coincident with apical F-actin (denoted by arrows in G, N and U). (Mag bar=20μm; n=5)

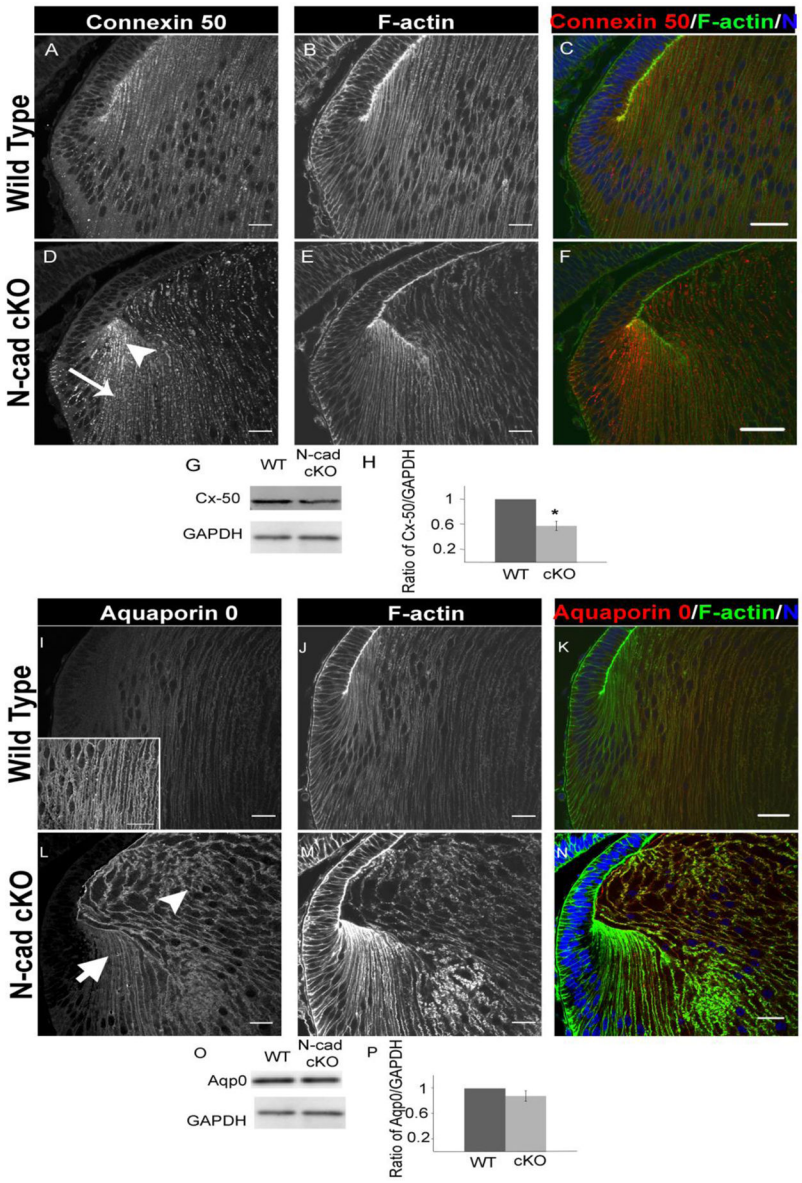

Figure 7. Connexin-50 and Aquaporin-0 are affected by loss of N-cadherin

Cryosections of E16.5 wild-type (A–C,I–K) and N-cadcKO (D–F, L–N) eyes were labeled for Connexin-50 (Cx50) (A,C,D,F), Aquaporin-0 (Aqp0), (I,K,L,N),F-actin (B,C,E,F,J,K,M,N), nuclei(C,F,K,N). Immunolocalization for Cx50 in N-cadcKO lenses showed that it was elevated at the apical tips of the newly differentiating secondary fiber cells adjacent to the lens fulcrum (D, arrowhead, and F), and had an stippled appearance along these cells lateral domains (D, arrow). Immunoblot analysis (G,H) at E16.5 showed decrease expression of Cx50 protein in the N-cadcKO lens. Immunolocalization of Aqp0 is highly up-regulated at fiber cell interfaces in N-cadcKO lenses (L,N) compared to wildtype lenses at the same settings (I,K). A higher intensity image of Aquaporin-0 in wildtype E16.5 lenses is depicted to demonstrate distinct localization of Aqp0 at cell-cell interfaces of normal fiber cells (I, inset). However, immunoblots for Aqp0 in E16.5 lenses show no

significant change in Aqp0 protein expression in the absence of N-cadherin, GAPDH included as control (O,P). (Mag bar=20μm; n=6; p<0.05*;p<0.01**; p<0.001***)

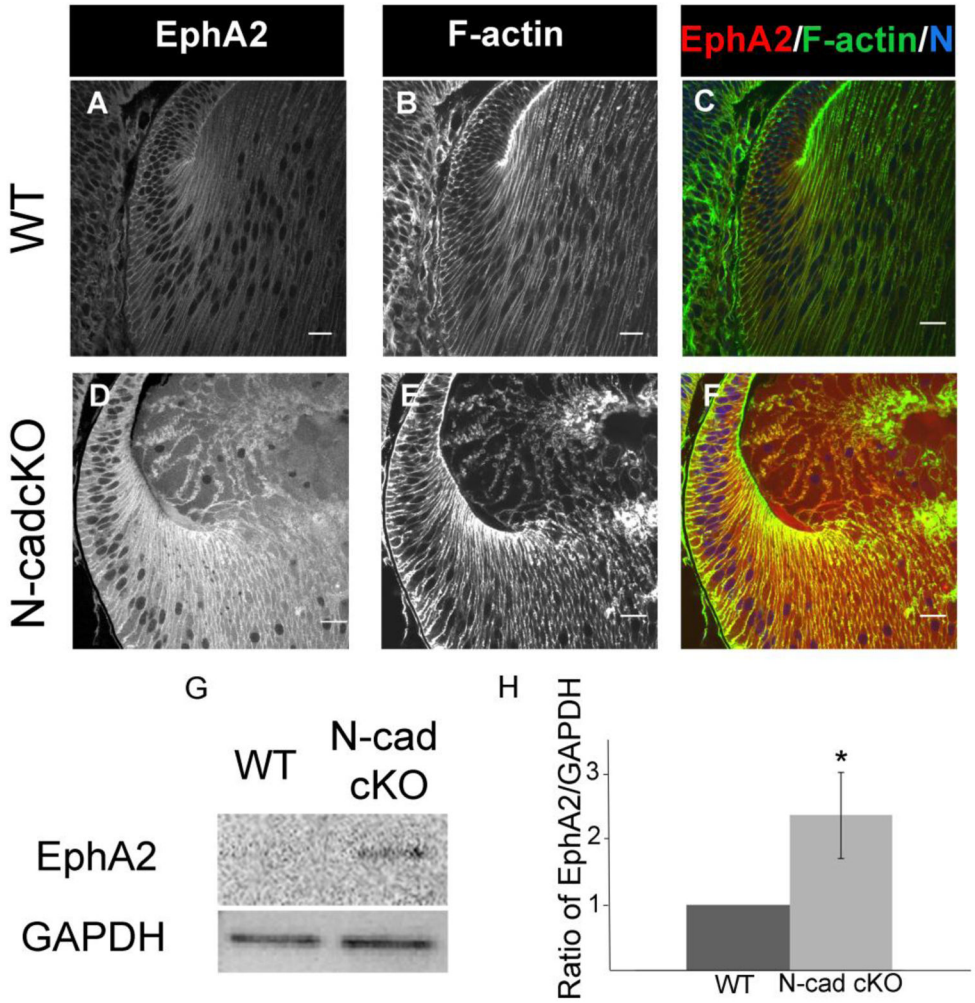

Figure 8. Loss of N-cadherin induces EphA2

Cryosections of E16.5 wild-type (A–C) or N-cadcKO (D–F) eyes were labeled for EphA2 (A,C,D,F), F-actin (B,C,E,F), and nuclei (C,F), denoted as N. There is considerable up-regulation of EphA2 at cell-cell borders throughout the N-cadcKO lens (D,F). E16.5 wildtype and N-cadcKO lenses were also immunoblotted for EphA2, with GAPDH as loading control (G,H) demonstrating significantly higher protein levels of EphA2 in N-cadcKO lenses (H). (Mag bar=20μm; n=4; $p<0.05$*;$p<0.01$**; $p<0.001$***)

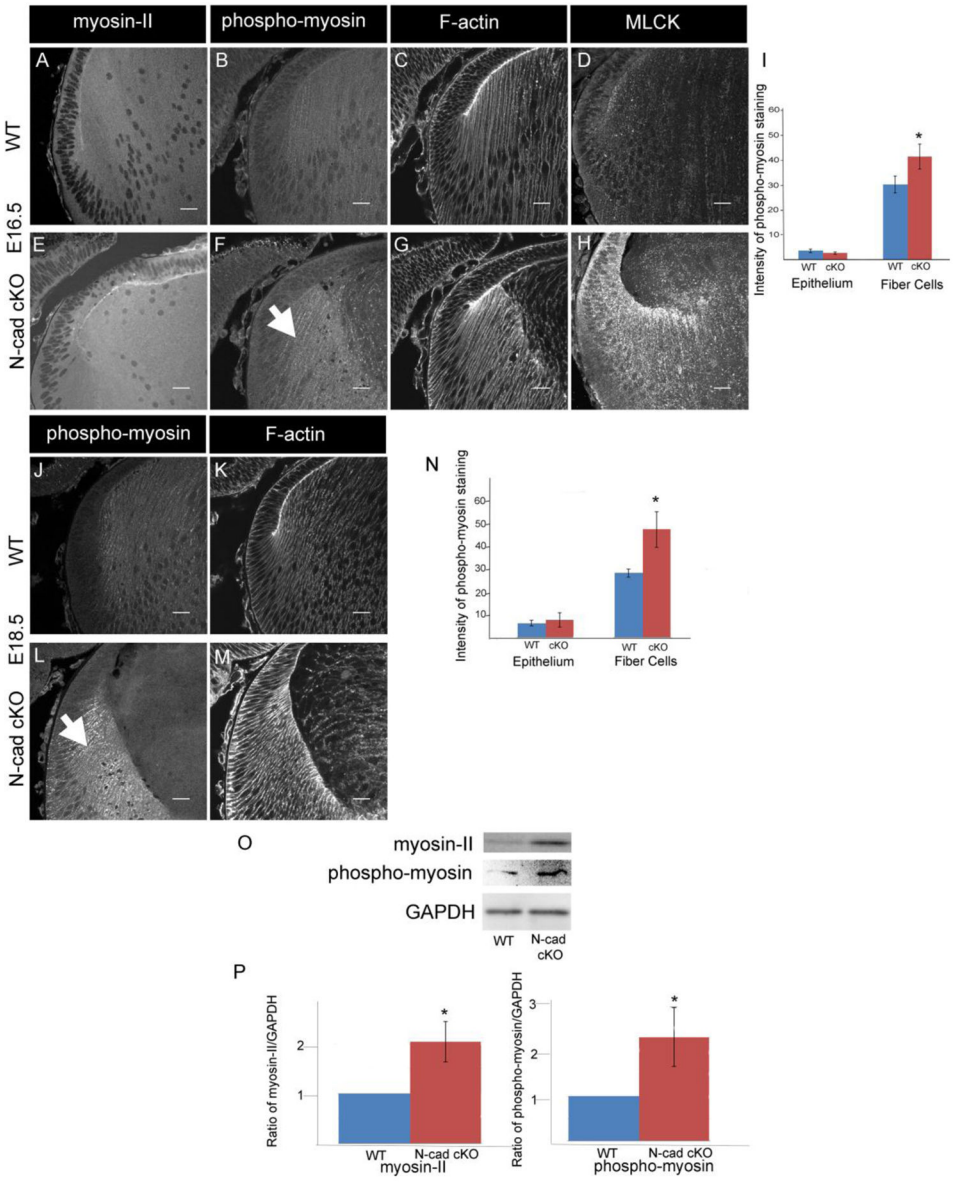

Figure 9. Increased expression of myosin light chain kinase (MLCK) and activation of myosin II in N-cadcKO lenses

Cryosections of wild-type (A–D,J–K) or N-cadcKO (E–H,L–M) eyes at E16.5 (A–H) and E18.5 (J–M) were labeled for myosin-II (A,E), phospho-myosin (B,F,J,L), myosin light chain kinase (MLCK) (D,H), F-actin (C,G,K,M). E16.5 lenses were immunoblotted for myosin-II and dual phospho-myosin, with GAPDH as control (O), and quantified (P). Myosin II protein levels were increased in N-cadcKO lenses (O,P), but myosin II localization was unchanged (E). In contrast, increased activation of myosin in N-cadcKO lens fiber cells was shown at both E16.5 and E18.5 by immunofluorescence (F,L, arrows) and immunoblotting (O,P) and is correlated with induction of MLCK (H). Histogram analysis of immunofluorescence intensity in epithelium and fiber cells at E16.5 (I) and E18.5 (N) demonstrated that the increased intensity of phospho-myosin immunolocalization

was fiber-cell specific and significant. (Mag bar=20μm; n=5 for immunolocalization; n=4 for immunoblot analysis; $p<0.05$*;$p<0.01$**; $p<0.001$***)

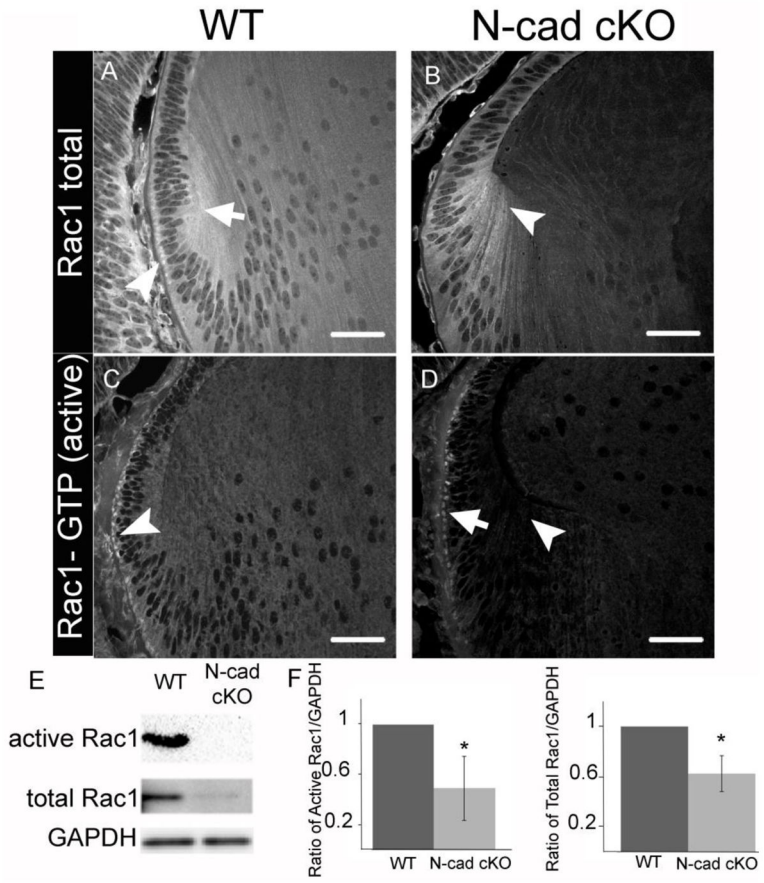

Figure 10. Rac 1 activity is lost in newly differentiating secondary fiber cells in the absence of N-cadherin

Cryosections of E16.5 wild-type (A,C) or N-cadcKO (B,D) eyes were labeled for total Rac1 (A,B) or active Rac1 (Rac-GTP; C,D). Rac1 localizes throughout the wild-type embryonic lens, highest in newly differentiating secondary fiber cells (A, arrow), and along the cells' basal surfaces (A, arrowhead). In the N-cadcKO lens Rac1 protein is still expressed only in lens epithelial cells and newly differentiating secondary fiber cells (B, arrowhead). Rac is activated throughout the normal lens (C), and is highest along the cells' basal surfaces (C, arrowhead). Rac activity is suppressed throughout the N-cadcKO lens (D), including the elongation-defective secondary fiber cells just past the lens fulcrum that retain expression of Rac1, but maintained along the cells' basal surfaces that are rich in integrin junctions (D, arrow). These results were confirmed by immunoblotting for both total and active Rac1 levels (E), which demonstrated that both total and active Rac1 are significantly decreased in N-cadcKO lenses (F). (Mag bar=20μm; n=5; $p<0.05$*;$p<0.01$**; $p<0.001$***)